

Review

Antibody-Drug Conjugates Targeting Tumor-Specific Mucin Glycoepitopes

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Abstract

Finding the ideal epitope to target is a key element for the development of an antibody-drug conjugate (ADC). To maximize drug delivery to tumor cells and reduce side effects, this epitope should be specific to cancer cells and spare all normal tissue. During cancer progression, glycosylation pathways are frequently altered leading to the generation of new glycosylation patterns selective to cancer cells. Mucins are highly glycosylated proteins frequently expressed on tumors and, thus, ideal presenters of altered glycoepitopes. In this review, we describe three different types of glycoepitopes that are recognized by monoclonal antibodies (mAb) and, therefore, serve as ideal scaffolds for ADC; glycan-only, glycopeptide and shielded-peptide glycoepitopes. We review pre-clinical and clinical results obtained with ADCs targeting glycoepitopes expressed on MUC1 or podocalyxin (Podxl) and two mAbs targeting glycoepitopes expressed on MUC16 or MUC5AC as potential candidates for ADC development. Finally, we discuss current limits in using glycoepitope-targeting ADCs to treat cancer and propose methods to improve their efficacy and specificity.

Keywords: glycoepitope; mucin; antibody-drug conjugate; cancer treatment

1. Introduction

With an aging global population, cancer incidence is on the rise, and, in most developed countries, cancer is the leading cause of death [1]. Conventional cancer therapies include surgery, radiotherapy, and chemotherapy [2]. While for many patients, these therapeutic interventions can effectively control tumor growth and there have been great strides in tailoring these to the molecular subtypes of neoplasia, there remains a large proportion of cancers that are refractory to treatment. Metastatic disease remains as a leading cause of cancer deaths with very few therapeutics that target this systemic phase of the disease [2]. Even when successful, chemotherapy-associated toxicity can often have a major impact on patient quality of life and drug-resistant relapse is common [3,4]. Antibody-based targeting of tumors offers more efficacious treatments with fewer adverse side effects. One of the ways to do this is using specific antibodies to tumor-restricted antigens to direct toxic payloads to the tumor while sparing normal tissue. In this review we will define such “tumor antigens” as antibody targets (rather than T cell receptor peptide antigens).

Antibody-drug conjugates (ADC) represent a relatively new class of cancer treatments that seek to avoid the off-target toxicity associated with chemotherapy by linking a cytotoxic drug (“payload”) to a tumor reactive monoclonal antibody (mAb) [5,6]. The goal of this site-specific delivery method is to minimize systemic chemotherapy-associated side effects and maximize delivery of the cytotoxic agent to tumor cells [5,6]. Ideal payloads are ones

with limited toxicity in the circulation while covalently linked to an antibody but that are highly toxic when internalized and released intracellularly. Several factors such as linker properties, drug-antibody-ratio, stability and biodistribution, and drug dosing, dictate clinical success of an ADC [7]. Of course, the selection of the target antigen and the mAb tissue/tumor specificity are crucial elements for the efficacy of this approach.

Ideal ADCs target an epitope highly expressed on cancer cells but absent or weakly expressed on normal cells [7]. Furthermore, an effective ADC epitope should be expressed on the surface of the cell (extracellular) and become internalized upon mAb binding [8]. Most ADC targets currently approved or in development are “tumor-associated” instead of “tumor-specific”, as they are also weakly expressed on normal tissue [9]. One interesting example of a true tumor-specific target is the epidermal growth factor receptor (EGFR) variant III (EGFRvIII) with deletions in *EGFR* exons 2–7. EGFRvIII is a tumor neoantigen that has attracted many efforts to generate mAbs, ADCs, vaccines, and chimeric antigen receptor T cell (CAR-T) candidate therapies [10,11]. Due to the important list of requirements for the generation of an optimal ADC, and primarily the scarcity of true tumor specific epitopes, optimal targets are extremely rare.

Intriguingly, the dysregulation of glycosylation pathways is a frequent feature of tumor progression as the resulting altered glycan structures participate in an array of biological processes involved in cancer development [12]. One



Table 1. Cancer-associated mucins.

Mucin	Normal tissue expression	Tumor expression pattern
MUC1	Glandular or luminal epithelial cells of the mammary gland, esophagus, stomach, duodenum, pancreas, uterus, prostate, and lungs, and to a lesser extent hematopoietic cells [22–24]	Breast, lung, endometrium, endocervix, ovary, bladder, kidney, esophagus, stomach, pancreas, colon and bile duct carcinomas [25,26]
PODXL	Kidney glomeruli, surface of vascular endothelial cells, megakaryocytes and platelets, mesothelial cells, hematopoietic progenitors, and a subset of neurons [27–32]	Embryonal, oral squamous, esophageal, lung, gastric, colorectal, pancreatic, prostate, bladder, thyroid, uterine and renal cell carcinoma as well as astrocytoma and glioblastoma [33,34]
MUC16	Epithelial cell surface lining the upper respiratory tract, cornea and conjunctiva, female reproductive organs, the pleura, the peritoneum, and the pericardium, the abdominal cavity, and the cervical mucus [35,36]	Pancreatic, esophageal, gastric, colorectal, breast, ovarian and non-small-cell lung cancers [35,37–39]
MUC5AC	Goblet cells in the lung, eyes, stomach and endocervix [40,41]	Pancreatic, colorectal, esophageal, gastric, mucinous ovarian and bronchoalveolar cancers [41–43]

important consequence of this dysregulated glycosylation is the generation of tumor-associated carbohydrate antigens (TACA) that comprise an array of potential tumor-specific neo-epitopes which, in theory, represent ideal ADC targets [13]. Mucins are a family of highly glycosylated extracellular proteins of particular interest as several members of this protein family are abundantly overexpressed on the extracellular surface of cancer cells, likely reflecting their role in altering adhesion and facilitating migration [14,15]. In this review, we provide an overview of the mechanisms that lead to TACA expression on mucins, the type of glycoepitope suitable for ADC-targeting and summarize results of select pre-clinical and clinical mucin-targeted ADC candidate therapies.

2. Mucins in Cancer

2.1 Mucin Overexpression in Cancer

Mucins are typically large glycoproteins (200 kDa–200 MDa) expressed by epithelial cell membranes [16]. This protein family is characterized by the presence of one or more modular mucin proline (Pro), threonine (Thr), serine (Ser) (PTS)-rich domains with a high frequency of Thr or Ser amino acid residues covalently conjugated with an α -N-acetylgalactosamine (α -GalNAc) moiety (*O*-linked glycosylation) [17,18]. The presence of the Pro interrupts the alpha-helix architecture allowing for an extended secondary structure with accessible Ser/Thr sites for glycan addition and, perhaps, display of glycans emanating radially from the protein core [19]. Although the vast majority of mucin glycans are of this *O*-linked glycan type, most also contain *N*-linked glycans through additional covalent modification of asparagine (Asn) residues [15]. Glycans often comprise more than 80% of the molecular mass of mucin molecules and this hydrophilic carbohydrate structure is thought to play key roles in modifying the biophysical properties of cellular membranes as well as alter their topology [16,20]. Following their complex synthesis in the cis and trans Golgi, mucins can either be secreted or anchored into

the plasma membrane via protein transmembrane domains or posttranslational attachment of lipids [15].

Mucins are frequently expressed, shed (proteolytic cleavage) or secreted on the apical surface of barrier structures of tissues, especially epithelial barriers contacting the extra-tissue environment (mucosal surface epithelium) and endothelial layers contacting the circulation [18]. Their presence maintains the integrity of these barriers while also limiting contact with pathogens, toxins and antigens and inflammatory agents that may cause damage and trigger inflammatory responses. Secreted/shed mucins can help neutralize pathogens and transmembrane mucins play a role in sensing the environment and maintaining tissue architecture. Tumorigenesis exploits the function of mucins (stochastically) to promote their own growth and survival; enhance motility; limit adhesion; and evade immune surveillance [18]. In addition, dysregulated expression of mucins during tumor progression may be accompanied by changes to glycosylation and metabolic machinery that result in aberrant mucin glycoforms with altered physical and functional properties including immune evasion [18,21]. In this review, we will selectively focus on four mucins that have been explored extensively for their role in cancer: transmembrane mucins Mucin 1 (MUC1), podocalyxin (Podxl), and MUC16 and secreted mucin MUC5AC (Table 1, Ref. [22–43]).

MUC1 is the founding member of large family of mucins normally expressed on glandular or luminal epithelial cells in a variety of tissues, where its extended negatively charged sugar branches create a selective biophysical barrier with anti-adhesive properties, limiting pathogen accessibility and preventing colonization of mucosal surfaces [23]. MUC1 has proved to be an interesting target for ADC development as aberrantly glycosylated MUC1 is overexpressed in most human epithelial cancers [25]. Podxl is a highly glycosylated cell surface sialomucin of the CD34 family of stem cell antigens and plays important roles in cell adhesion and transendothelial migration in normal and cancer tissues [44,45]. Its expression is upregulated by a wide

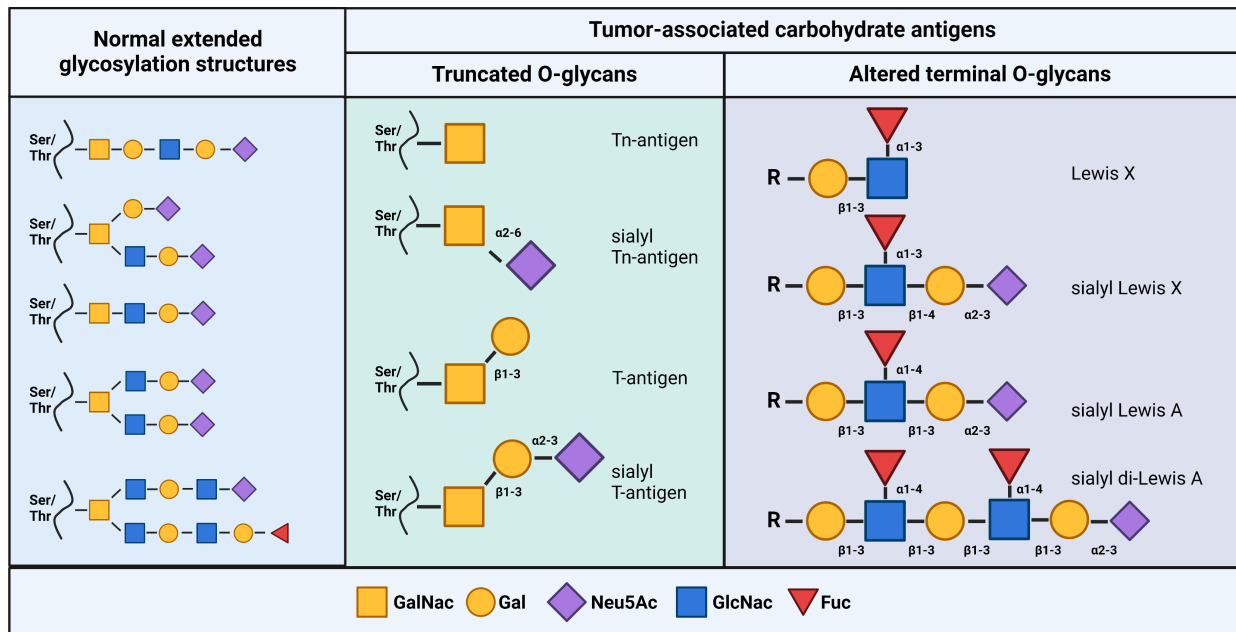


Fig. 1. Glycan structures of tumor-associated carbohydrate antigens. (Left) Examples of extended glycans expressed on normal cells. (Middle) Examples of common truncated *O*-linked glycans. (Right) Examples of altered terminal *O*-linked glycans. Created with BioRender.com.

variety of cancer types and Podxl overexpression is consistently linked to poor prognosis, more aggressive tumor progression, unfavorable treatment outcomes, and possibly chemoresistance [32–34]. In keeping with its normal distribution on hematopoietic and early embryonic tissue stem cell populations, Podxl appears to be expressed by a highly mobile subset of tumor initiating cells. Furthermore, inactivation of the *PODXL* gene or dampening of its expression in cell lines cripples their ability to form tumors in xenografts and dampens their “tumorsphere” forming potential *in vitro* [46,47]. MUC16, a MUC1 relative, is the largest of all known mucins and is the carrier of the Cancer Antigen 125 (CA125) epitope, which is widely used as a serum marker for the detection of ovarian cancer [35,48,49]. MUC5AC is a secreted, gel-forming, mucin that normally composes the airway mucus layer [50,51]. In cancer, its expression is upregulated by an array of tumor types and its expression is predominantly cytoplasmic or at the apical pole of tumor cells [41].

2.2 Dysregulation of Mucin *O*-Linked Glycosylation in Cancer

O-linked glycosylation of mucins is initiated with the monosaccharide GalNAc α -linked primarily to Ser/Thr residues. This *O*-linked GalNAc is then further extended by addition of different monosaccharides catalyzed by 30 or more distinct glycosyltransferases (GTF) [52]. In contrast to *N*-linked glycosylation which occurs co-translationally in the endoplasmic reticulum (ER), *O*-linked glycan post-translational modifications are initiated in the Golgi apparatus and occur after most key protein folding events have

taken place [52]. Several mechanisms can contribute to the alterations in *O*-linked GalNAc glycosylation observed in cancer including changes in expression or localization of GTFs, altered expression or activity of glycosidases (enzymes responsible for the hydrolysis of glycosidic linkages), changes in pH of the Golgi apparatus, modifications in the identity and concentration of UDP-sugar donors, and finally, large alterations in the abundance of the target mucin substrates (apomucins) themselves [53,54]. These alterations produce three classes of TACAs: (1) oncofetal antigens that are rare in normal adult tissue but commonly expressed in fetal tissue; (2) neoantigens expressed only in tumor cells; and (3) altered levels of normal antigens [54]. Oncofetal antigens and neoantigens are ideal targets for ADCs as they are typically tumor-restricted in adults. These oncofetal and neoantigen TACAs can be either truncated *O*-linked glycans that reveal hidden or cryptic antigens (Tn-, sTn- or T-antigens) or altered terminal glycans (Lewis system carbohydrates, for example) (Fig. 1) [54,55]. While the focus of this review is on mucin glycoepitopes, other tumor-expressed glycoproteins also display TACAs. One good example is the carcinoembryonic antigen (CEA) or CECAM5, which is a glycoprotein member of the immunoglobulin family and one of the first described tumor antigens [56]. Although originally thought to be an embryo-restricted marker that is (re)expressed on tumors, CEA is also found on normal mucosal tissue in adults [57]. However, its glycosylation profile is altered on cancer cells with increased expression of Lewis X and Lewis Y motifs [58,59].

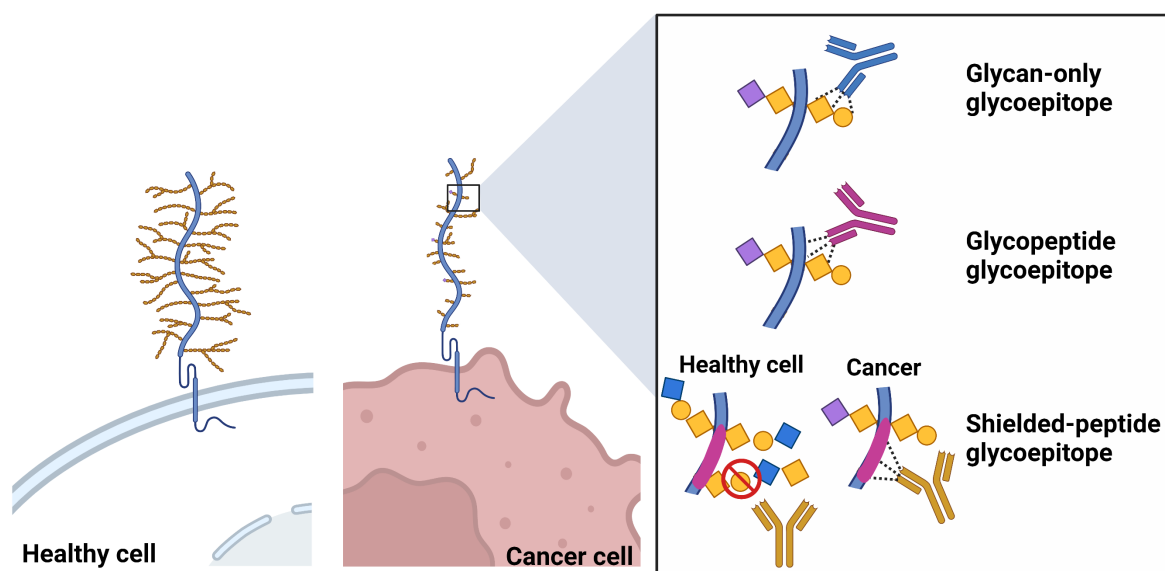


Fig. 2. Schematic representation of glycan-only, glycopeptide and shielded-peptide glycoepitopes. Created with BioRender.com.

2.3 Mucin Epitopes Arising from an Altered Glycome

By definition, a glycoepitope refers to a carbohydrate moiety that is recognized by a mAb or other glycan-binding protein [60]. While mAbs that only bind monosaccharide units (glycan-binding mAbs) exist, their affinity is usually much lower than protein-specific mAbs with equilibrium dissociation constant (K_D) values in the micromolar (μM) range compared to nanomolar (nM) range, respectively [61,62]. Since glycan epitopes are frequently repeated on a protein core, especially in the case of mucins with multiple tandem glycosylation targets, low affinity of glycan-binding mAb can be circumvented by generating mAbs with two or more binding sites that recognize repeating glycan-epitope to form a multivalent complex [63]. This is reflected by their early expression as deca-valent IgM and their observed class switching bias toward IgG₃ in mice and IgG₂ in humans—mAb isotypes that tend to self-associate through their constant regions to form multivalent networks [61,64,65]. As an alternative approach, mucins may accommodate bi-paratopic mAb that recognize two different nonoverlapping epitopes on the same antigen to improve glycan-binding mAb avidity. Bi-paratopic ADCs have superior internalization profiles compared to monospecific and bispecific counterparts (reviewed in ref [66]).

Another class of TACA-binding mAbs that, in general, tend to exhibit improved binding affinities and specificity are those mAbs that recognize a glycopeptide formed by combinations of a glycan and a defined peptide epitope (i.e., glycopeptide glycoepitopes) [67,68]. These tend to show specificity and affinities more typically associated with protein antigens. A third type of glycoepitope that we have named “shielded-peptide glycoepitopes” are not true glycoepitopes *per se* as the mAb does not directly bind the gly-

can, but instead recognize polypeptide epitopes whose accessibility to the peptide sequence is restricted by the altered glycosylation status of the glycoprotein expressed on normal cells. A schematic representation of these three classes of epitopes is presented in Fig. 2.

While the identification of the amino acid sequence of a regular peptide epitope is now relatively easy, the characterization of a glycoepitope structures remains complex and challenging due to their complex and branched chain structure. For this reason, the exact glycoepitope structure of most mAb/ADC targets discussed in this review is still unknown. Nevertheless, we have included mAbs/ADCs with sufficient epitope mapping data to suggest that their epitope falls within one of three categories described in Fig. 2.

3. ADCs Targeting Tumor-Specific Mucin Glycoepitopes

Therapeutic mAbs and associated ADCs included in this review (summarized in Table 2, Ref. [69–106]) were selected to provide illustrative examples of tumor-specific mucin glycoepitopes that may be ADC targets. As the glycoepitope nature of the target is the central focus of this review, several interesting anti-mucin ADCs were excluded due to the peptide nature of their epitope or the lack of sufficient epitope mapping information to support the contention that their epitope falls within one of the three types of glycoepitopes described in Fig. 2.

It is difficult to compare mAb affinities across examples especially when disparate methods of assessment are used between different research groups. While still imperfect, here we report antibody-antigen affinity values (K_D – dissociation constant) when the assessment was performed on epitope-expressing live cells (Scatchard analysis or KinExA methods unless otherwise indicated).

Table 2. Antibody and antibody-drug conjugates targeting tumor specific mucin glycoepitopes.

I. Antibodies to unknown glycan epitopes			
Antibody/Target	ADC	Epitope expression	Pre-clinical/clinical development stage
mJAA-F11 huJAA-F11 H2aL2a	huJAA-F11 H2aL2a-DM1	Targets glycans of an <i>O</i> -linked glycosylated protein expressed in breast, lung, prostate, colon, bladder, and ovarian tumors [69,70]	<i>In vitro</i> , <i>in vivo</i> mouse models [71]
FG129 (m) CH129 (ch IgG ₁) [72]	CH129-DM1 CH129-DM4 CH129-MMAE	Putative mucin expressed glycans in pancreatic, gastric, colorectal, ovarian, and NSCLC tumors	<i>In vitro</i> and <i>in vivo</i> mouse models
II. Antibodies binding glycoepitope-dependent epitopes of MUC1			
Antibody	ADC	Epitope expression	Pre-clinical/clinical development stage
mAb 16A	16A-MMAE	Breast tumor, NSCLC and gastric tumors [73,74]	<i>In vivo</i> mouse models [74]
PankoMab (m) Gatipotuzumab/PankoMab-GEX (hu)	β -amanitin-conjugated PankoMab	Cervical, ovarian, lung, breast, gastric, colorectal, liver, kidney, and thyroid tumors [75–78]	β -amanitin—PankoMab <i>in vitro</i> [79] gatipotuzumab /PankoMab-GEX humanize unconjugated form Phase I (NCT0122624) [80,81] Phase II (NCT01899599) [82,83]
(m)DS6 (hu)DS6	huDS6-SPDB-DM4 (SAR566658)	Breast, ovary, lung, bladder, and pancreas tumors [84–86]	<i>In vivo</i> mouse models [86] Phase I (NCT01156870) [87] Phase II (NCT02984683) (discontinued) [88]
C242 (m) huC242 or cantuzumab (hu)	huC242-DM1 (cantuzumab mertansine) huC242-DM4 (cantuzumab ravtansine/IMGN242)	Pancreas and colorectal tumors [89–91]	<i>In vivo</i> mouse models (DM1) [92,93] (DM4) [94] huC242-DM1 Phase I [95–97] huC242-DM4 Phase I [98] Phase II [99]
III. Antibodies to non-MUC1 glycoepitopes			
Antibody	ADC	Epitope expression	Pre-clinical/clinical development stage
PODO447	huPODO447-Vedotin (MMAE)	Tumor specific glycoform of PODXL expressed in ovarian tumors [47]	<i>In vitro</i> and <i>in vivo</i> [47,100]
AR9.6	ADC not yet developed	MUC16 expressed in pancreatic tumors [101]	unconjugated mAb, <i>in vivo</i> mouse models [101,102]
NPC-1 NPC-1C ensituximab (NEO-102)	ADC not yet developed	MUC5AC expressed in colon and pancreatic tumors [103]	unconjugated mAb, <i>in vivo</i> mouse model [103] Phase I [104] Phase II [105] Phase I/II (NCT01040000) [106]

m, mouse; hu, humanized; rb/hu, rabbit/human chimera; NSCLC, non-small cell lung carcinoma; ch, chimeric human; MMAE, monomethyl auristatin E; DM1, maytansinoid DM1/mertansine; DM4, maytansinoid DM4.

3.1 Glycan-Binding ADCs

A prime example of a glycan-binding mAb is the murine JAA-F11 IgG₃ or humanized hJAA-F11 H2aL2a IgG₁ that binds the T-antigen (sometimes call a Thomsen-Friedenreich (TF)-antigen) formed by the disaccharide, D-galactose-beta-(1-3)-N-acetyl galactosamine (Gal-β-(1-3)-GalNAc), alpha (α)-linked to Ser/Thr peptide residues [69,71] (Fig. 1, Table 2). The T-antigen is a cryptic oncofetal antigen frequently expressed by mucins in cancer [107]. In contrast to other T-antigen targeting mAbs, the JAA-F11 mAbs are highly specific for the alpha-linked tumor-associated T-antigen and not the beta-linked structure expressed on the surface of normal tissues [69,71]. JAA-F11 mAbs are excellent candidates for ADC development as they are rapidly internalized upon binding their ligand [71,108]. Indeed, when conjugated to the microtubulin inhibitor DM1 (N²'-deacetyl-N²'-(3-mercapto-1-oxopropyl)-maytansine), the hJAA-F11 H2aL2a-DM1 ADC demonstrated *in vitro* cytotoxic activity against various triple negative human breast cancer and lung cancer cell lines and significantly reduced MDA-MB-231 tumor growth in a mouse xenograft model [71]. This ADC has the potential to treat a significant number of patients, given the widespread expression of the T-antigen (up to 80% positive breast tumors) and its expression by a variety of cancer types including breast, lung, prostate, colon, bladder, and ovarian cancers [69,70]. Although the ability to kill xenografted tumors appears quite promising, this effect required an intense dose regimen (15 mg/kg 3 times a week for the first week followed by weekly injections for 5 weeks) and the study did not include a non-targeting ADC raising concerns of the specific efficacy of antibody-mediated tumor killing in this study [71].

Another interesting glycan-binding mAb is the mouse IgG₁ FG129 and its chimeric human IgG₁ variant, CH129 (Table 2). These recognize, with high affinity (KinExA $K_D \sim 21\text{--}59$ nM [72]), terminal sialyl-di-Lewis^a (s-di-Le^a) glycans as well as the two closely related glycans, sialyl-Lewis^a-Lewis^x (sLe^a/sLe^x) and sialyl-Lewis^a (sLe^a) expressed on several high molecular weight glycoproteins (likely mucins) (Fig. 1) [72]. *In vitro* studies with colorectal and pancreatic cancer cell lines demonstrate that CH129 mAb binding to the cell surface of tumors drives epitope internalization. Conjugation with three different linker-payloads (monomethyl auristatin E (MMAE) or two maytansinoids (DM1 and DM4)) leads to higher cytotoxicity compared to their respective non-targeting rituximab (RTX) control ADCs. CH129 coupled to MMAE (CH129-MMAE) showed an impressive half-maximal effective concentration (EC₅₀) in the picomolar (pM)-to-nM range and its non-targeting control RTX-MMAE showed no cytotoxicity. Using an *in vivo* evaluation in a COLO205 xenograft mouse model, CH129-MMAE conjugate exhibits potent control of tumor growth and elimination of tumors in 7 of 10 mice for the duration of the study with a therapeutic dose

of 5 mg/kg (biweekly) [72]. The CH129 glycoepitope is expressed on a variety of cancer types suggesting a broad potential for therapeutic interventions. While this epitope is mostly tumor-specific, FG129 binds weakly to a small percentage of cells within gallbladder, ileum, liver, esophagus, pancreas, and thyroid tissues and therefore off-target effects during clinical development remain a potential concern [72].

3.2 MUC1 Glycoepitope Binding mAbs

Several MUC1-directed mAbs and single-chain variable fragments (scFv) have been generated as potential cancer therapeutics for CAR-T, radioimmunotherapy (RIT) and mAb therapy that may have applications as ADCs (reviewed in ref [109]). These mAbs, as a collection, target glycan or peptide epitopes as well as glycoepitopes [109]. Below we highlight examples of established MUC1-directed ADC candidates that target glycoepitopes.

16A is a murine IgG₁ that targets a glycoepitope of MUC1 [73,74]. This mAb strongly binds to the glycopeptide RPAPGS(GalNAc)TAPPAHG, an aberrantly glycosylated tandem repeat region of MUC1 but displays much weaker binding (25-fold lower) to the non-glycosylated RPAPGSTAPPAHG peptide (relative affinity determined by ELISA) [73]. Interestingly, the affinity of 16A for the isolated peptide and glycosylated peptide measured by surface plasmon resonance (SPR) are comparable (SPR $K_D \sim 500\text{--}1000$ nM) [73]. The much higher apparent affinity for aberrantly glycosylated MUC1 on live cells and tumors might be due to conformational changes induced by the glycan that facilitates access of the mAb to its peptide epitope (i.e., a shielded-peptide glycoepitope) or, alternatively, 16A may engage in intermolecular contacts simultaneously with the peptide and the glycan moiety (i.e., glycopeptide epitope). The 16A mAb binds to target epitopes expressed on lung, breast (including triple-negative breast cancer) and gastric cancer tissues and is rapidly internalized supporting its further development as a therapeutic ADC candidate [74]. To test its potential, Pan *et al.* [74] generated the 16A-MMAE ADC and demonstrated that it potently kills lung, breast, pancreatic, gastric, and ovarian cell lines *in vitro*. Furthermore, using the adenocarcinoma H838 (non-small cell lung cancer) in a mouse xenograft model, they demonstrated that the 16A-MMAE ADC inhibits tumor growth in a dose dependent manner (minimum therapeutic dose ~ 5 mg/kg) [74]. However, the omission of a non-targeting ADC as a control in these experiments somewhat limits the ability to evaluate conclusions of its “on”, versus “off” target efficacy [74].

Gatipotuzumab, formerly known as PankoMab-GEX, is the humanized version of the mouse IgG₁ PankoMab. This mAb was initially generated to maximally discriminate between the carbohydrate-induced conformational tumor epitope on MUC1 (TA-MUC1) and the non-glycosylated MUC1 epitope [79]. The TA-MUC1 epitope includes

the ...PDT*RP... amino acid, where T* is O-glycosylated with GalNAc α 1- or a similar short, non-sialylated, glycan such as Gal β -(1–3)-GalNAc α 1- (core-1/T-antigen) [110]. The exact interaction sites between PankoMab and its epitope are unknown, but its strong binding with the glycosylated version of the TA-MUC1 peptide and weak binding with the non-glycosylated version of the same peptide strongly suggest that PankoMab epitope is a glycopeptide epitope [79]. Antibody affinity estimates range from ~1 to 7 nM (Scatchard K_D) for tumor cell lines [79]. Due to its antibody dependent cellular cytotoxicity (ADCC) activity [79], Gatipotuzumab (unconjugated form) was tested in Phase I clinical trials (NCT01222624) in patients with advanced TA-MUC1-positive carcinomas and Phase II trial (NCT01899599) in patients with TA-MUC1-positive ovarian tumors. While the mAb was well tolerated in ovarian cancer patients, unfortunately, gatipotuzumab did not improve disease outcomes compared to the placebo [81,83]. The potential of gatipotuzumab to function as an ADC, however, has not been fully explored. Its rapid internalization and its capacity to induce toxin-mediated antigen-specific tumor cell killing *in vitro* suggest it is a good candidate for further research [79]. Interestingly, in 2018, Daiichi-Sankyo (Japan) and Glycotope GmbH (Germany) entered into an exclusive worldwide licensing agreement to develop an ADC by combining Daiichi-Sankyo's proprietary ADC technology with Glycotope's gatipotuzumab [111].

The murine MuDS6 IgG₁ mAb was generated by Smith *et al.* in 1999 [112] in a screen to produce a mAb that reacts with an antigen that is tumor cell surface expressed but with limited expression on normal tissue. The resulting mAb was later humanized as huDS6 IgG₁ mAb [113]. The DS6 mAbs recognize a sialic acid-dependent epitope (sialoglycotope) of MUC1 designated "CA6" [84,86]. However, the exact glycopeptide structure remains to be determined and could be either a glycopeptide epitope or a shielded-peptide glycopeptide. The huDS6 mAb is efficiently internalized in an antigen dependent manner and has been conjugated to the cytotoxic maytansinoid derivative DM4 to generate SAR566658 (huDS6-SPDB-DM4 ADC) [86,114]. This ADC induces targeted *in vitro* cytotoxicity and effectively controls tumor growth in murine xenograft tumor models using a variety of human tumor cell lines. Importantly, the efficacy of SAR566658 is associated with high CA6 expression in tumor targets [86]. In a Phase I study in CA6-positive patients with metastatic breast cancer (NCT01156870), SAR566658 provided a favorable safety profile and encouraging antitumor activity [87,115]. However, a subsequent Phase II study in metastatic triple-negative breast cancer patients (NCT02984683) was discontinued following preliminary analyses showing that the benefit/risk balance was not favorable due to a higher-than-expected incidence of ophthalmologic events (e.g., keratitis and keratopathy) [86,88]. While DS6 mAbs predom-

inantly bind to tumor tissues, they also recognized some normal adult tissues (e.g., fallopian tube, pulmonary alveoli and urothelium) and these could compromise its utility for tumor-specific targeting [84].

The C242 mouse IgG₁ mAb or its humanized version huC242 mAb (cantuzumab) recognize, with high selectivity, the extracellular CA242 epitope present on the cancer antigen (CanAg) glycoform of MUC1 [91,116,117]. The exact structure of the epitope, too, has not been determined but, because it contains a sialic acid, it is likely one of the three classes of glycopeptides described in this review [116]. First demonstrated in 1996 by Liu *et al.* [92], the murine C242 mAb combined with the maytansinoid DM1 (C242-DM1) can effectively eradicate CanAg-expressing human tumor xenografts in mice in an antigen-specific manner. The capacity of its humanized version (huC242-DM1) to eliminate CanAg-positive COLO205 xenograft tumors was later confirmed and it was also demonstrated that this ADC can induce an interesting bystander effect and kill the proximally located antigen-negative tumor cells [93]. The therapeutic potential of the huC242-DM1 ADC (SB-408075: cantuzumab mertansine) was tested by ImmunoGen (USA) in partnership with GlaxoSmithKline (UK) in a Phase I clinical trial to determine the optimal treatment schedule and the limiting dose toxicity [95–97]. Patients with CanAg-expressing solid malignancies were included in these trials and early signs of activity of huC242-DM1 in tumors with strong intensity of CanAg was observed. However, dose-related hepatotoxicity halted further dose escalation and therapeutic trials and development was discontinued [118]. Evaluation of a derivative of huC242 conjugated to DM4 (IMGN242: cantuzumab ravtansine) caused complete tumor regression in mice bearing human gastric carcinoma xenografts [94]. IMGN242 was well tolerated in a Phase I clinical trial (NCT00352131) [119]. In a Phase II trial (NCT00620607) [120], in 6 patients with CanAg-positive gastric or gastroesophageal junction cancer, a partial response was observed in 1 patient. Unfortunately, 3 out of 6 patients developed ocular toxicities that were assessed to be study drug related [99] and the huC242-DM4 program was later discontinued [118].

While MUC1 glycopeptides are interesting targets for the development of ADCs, the few targeting mAbs that reached the clinical trial stage have, to date, failed to be approved for cancer treatment. Possible explanations for these disappointing results will be discussed at the end of this review.

3.3 Glycopeptide Binding mAbs Targeting Other Mucins

Abnormal mucin-glycosylation patterns in cancer are not restricted to MUC1. We recently developed a highly tumor specific rabbit/human chimeric IgG₁ mAb, named PODO447 that reacts with Podxl expressed on tumor cells but not normal tissue [47]. This mAb specifically binds a glycopeptide epitope consisting of the core 1 O-linked gly-

can (T-antigen) in the context of the Podxl polypeptide but does not recognize general core 1 glycans decorating other proteins [100]. The PODO447 affinity for human tumor cell lines is well into the sub-nanomolar range (KinExA K_D 8 to 190 pM) [121]. PODO447 is effectively internalized in cancer cells and, when conjugated to a protease-cleavable linker with MMAE (vedotin), PODO447-ADC induces *in vitro* cytotoxicity in variety of cancer cell lines in an antigen-dependent manner [47]. *In vivo*, PODO447-ADC treatment leads to regression of xenografted human ovarian and pancreatic tumors with a therapeutic dose of 2–4 mg/kg [100]. Further development of this promising ADC awaits Phase I and Phase II trials.

AR9.6 is a murine mAb that binds a conformational epitope of the SEA domain 5 of MUC16 that is influenced by *O*-linked glycosylation [101]. Interestingly, binding of unconjugated AR9.6 with MUC16 leads to a significant reduction of tumor growth in a pancreatic tumor model by blocking MUC16-dependent activation of ErbB (EGF) receptors on the cancer cell surface and downstream attenuation of oncogenic AKT and GSK3 β signaling [101]. This mAb has also been conjugated to a near-infrared fluorescent dye and radiolabeled with [⁸⁹Zr]Zr⁴⁺ to enable PET-imaging. In both cases, these AR9.6 conjugations lead to MUC16-dependent tumor targeting in different *in vivo* cancer models [102,122]. Good target-mediated internalization of a humanized AR9.6 by tumor cells suggests promising ADC therapeutic potential for this mAb [102,122].

Finally, NEO-102 (ensituximab) is a chimeric mouse/human IgG₁ mAb that recognizes the NPC-1C glycoepitope of MUC5AC and is described as an aberrantly glycosylated epitope preferentially expressed in pancreatic and colorectal cancers. NEO-102 can therefore discriminate between the native MUC5AC expressed on normal tissue and a variant of MUC5AC expressed by tumors [103,123]. The exact structure of the epitope remains to be identified, but its dependence on a tumor-specific glycosylation profile suggests it is one of the three classes of glycoepitope described in this review. In an *in vivo* mouse model, unconjugated NEO-102 significantly reduced human pancreatic CFPAC-1 tumor xenograft growth [103]. However, in a Phase II clinical trial [106] funded by Precision Biologics Inc (USA), NEO-102 only demonstrated modest antitumor activity in patients with refractory metastatic colorectal cancer [105]. Interestingly, the unconjugated mAb treatment was well-tolerated by these patients suggesting that linking NEO-102 to a cytotoxin to generate an ADC could safely increase the antitumor potential of NEO-102 [104,105].

4. Discussion

While most mucin glycoepitope-targeting ADCs described in this review were highly effective at eliminating tumor cells *in vitro* and in animal models, the few that reached clinical trials have so far failed to provide con-

vincing results and some have revealed unexpected side effects. These pitfalls are not unique to glycoepitope-specific antibodies and there are several factors that might explain these unfortunate results (summarized in Fig. 3). First, the shedding or secretion of the extracellular mucin domain containing the ADC glycoepitope can reduce the specific binding of the ADC to its target. This can simultaneously lead to off-target antigen-ADC interaction with cells that bind the shed antigen leading to toxicity while, at the same time, lessening the portion of the administered drug reaching the tumor microenvironment, reducing efficacy, and potentially leading to an unnecessary dose escalation to deliver drug on target [124–128]. With that in mind, careful measurement of the target glycoepitope entering the circulation during the clinical trials to assess potential of shedding to undermine efficacy could prove to be an important further criterion and evaluating potential of these types of targets in evaluating the potential of these types of targets. This approach was used in the IMGN242 trial to adjust dosing in patients with low CanAg circulating in plasma to mitigate off-target toxicity [99].

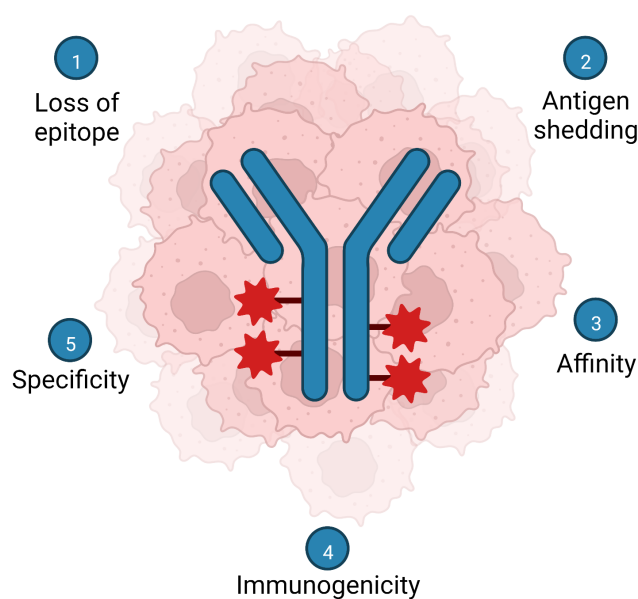


Fig. 3. Summary of notable challenges facing development of mucin glycoepitope therapeutic mAb and ADC. (1) Loss of the target epitope by the tumor and tumor heterogeneity. (2) Shedding or secretion of the epitope. (3) Carbohydrate mAbs tend to bind epitopes with lower affinities compared to peptide epitopes. (4) Carbohydrate epitopes are less immunogenic than peptide epitopes. (5) Identifying truly tumor-restricted mucin glycoforms (tumor antigens) Created with BioRender.com.

Another consideration should be the tumor specificity of the glycoepitope. While the expression of all the glycoepitopes targeted by the ADCs described in this review are predominantly present on cancer cells, many are also

expressed at lower levels on subsets of normal tissue cells [72,74,84]. Depending on the type of tissue and the level of non-tumor expression, this could lead to significant normal tissue toxicity. The cBR96-doxorubicin (BR96-DOX/SGN-15), a “first generation” ADC directed against a putative “tumor-specific” glycoepitope (Lewis Y), is a good example of how the expression of the ADC target on normal tissue can negatively influence its efficacy. This ADC failed clinical trials due, in part, to the expression of Lewis Y in gastrointestinal (GI) mucosa that caused both toxicity (adverse GI events) and poor therapeutic performance (an “antigen sink” that sequesters the drug) [129].

Glycan immunogenicity is generally inferior to peptide antigens and, therefore, the affinity of mAbs is stronger for peptide sequences compared to glycans [61]. Since much of the tumor specificity of the epitope is provided by the glycan structure, the optimal glycoepitope target is likely a glycopeptide epitope rather than one comprised purely from glycans. Shielded-peptide glycoepitopes are also good alternatives, but as several altered glycosylation profiles could provide mAbs with access to an otherwise, shielded-peptide epitope, the tumor-specificity might be compromised in some normal contexts. Currently, the characterization of the exact glycan structure of the epitope and the identification of the interactions between the mAb and the glycoepitope remain challenging to obtain. New technologies such as tandem mass spectrometry (MS/MS) used in combination with new computational tools will help predict the exact structure of glycoepitopes and might help select candidate mAbs that are more likely to be tumor-specific [130]. Likewise, the screening of cellular arrays bearing lesions in known GTFs (as was done for the PODO447 mAb [100]) could prove to be a highly effective method of fine mapping the glycoepitopes on target antigens.

Another factor that can limit the efficacy of mucin glycoepitope targeting ADCs is the possibility that, at some point during the treatment, tumors undergo selective pressure to alter their expression of GTFs or that existing variants lacking such enzymes expand when reactive subsets are eliminated. Either of these mechanisms would be expected to allow tumors to escape therapeutic targeting. To better characterize this phenomenon in mAb and target specific scenarios, the expression of the glycoepitope should be carefully monitored following the treatment in pre-clinical models and during clinical trials whenever possible. Furthermore, a potential therapeutic approach to circumvent this problem could be to combine the ADC treatment with other therapies, such as immunotherapies, to help eradicate cells negative for the ADC-glycoepitope [131]. In this regard, it is intriguing that preclinical studies with the PODO447-ADC targeting Podxl have shown that residual tumors remaining after ADC treatment fail to express the Podxl polypeptide rather than maintaining its expression and simply altering its glycosylation [100]. At face value,

this may suggest that the pattern of GTF expression by aggressive tumors plays a more critical role to their biology than expression of the individual polypeptide itself. More strikingly, the cells that lose expression of the Podxl core protein are known to grow more slowly and are less invasive. Finally, these residual Podxl-negative tumors appear to be more prone to clearance by the endogenous immune response (i.e., the ADCs are much more efficacious in Nude mice than in the more severely immunocompromised NSG mice) suggesting that killing of Podxl+ subset tumor cells may have an outsized effect on tumor clearance. These observations bode well not only for the PODO447-ADC but also for development of any other tumor-specific glycoepitope antibodies that detect a similar glycan-modification.

In conclusion, ADCs targeting mucin’s glycoepitopes represent a novel opportunity for highly selective tumor-targeting. While the ability to generate antibodies to identify, and fine map these target epitopes have all been challenging, once identified, the data suggest that these may prove to be highly specific at targeting the most relevant subsets of tumor cells that drive disease. The few emerging mucin targets described here may indeed be the vanguard of a much larger constellation of potential glycoepitopes. With that in mind, a well-developed, stepwise pathway for systematic evaluation of the tumor glycoepitope space could prove highly revealing of new actionable targets.

Author Contributions

JB realized the original draft preparation. JB and MRH designed the figures. MRH, CDR and KMM reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

MRH, CDR and KMM are co-inventors on PODO447 patent application(s) assigned to the Centre for Drug Research and Development University of British Columbia, the University of British Columbia and, the University of Copenhagen.

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